NOVEL METHODS FOR DISPLAYING (POLY)PEPTIDES/PROTEINS ON BACTERIOPHAGE PARTICLES VIA DISULFIDE BONDS

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a divisional of U.S. Application Serial Number 09/809,517, filed March 15, 2001, the entire contents of which are expressly incorporated herein by reference. This application is based upon, and claims priority to, European patent applications EP 99 11 4072.4 and EP 00 10 3551.8, which are incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

The present invention relates to methods for displaying (poly)peptides/proteins on the surface of bacteriophage particles by attaching the (poly)peptide/proteins via disulfide bonds. A number of documents are cited throughout this specification. The disclosure content of these documents is herewith incorporated by reference in their entirety.

Smith first demonstrated in 1985 that filamentous phage tolerate foreign protein fragments inserted in their gene III protein (pIII), and could show that the protein fragments are presented on the phage surface (Smith, 1985). Ladner extended that concept to the screening of repertoires of (poly)peptides and/or proteins displayed on the surface of phage (WO 88/06630; WO 90/02809) and, since then, phage display has experienced a dramatic progress and resulted in substantial achievements.

Various formats have been developed to construct and screen (poly)peptide/protein phage-display libraries, and a large number of review articles and monographs cover and summarise these developments (e.g., Kay et al., 1996; Dunn, 1996; McGregor, 1996).

Most often, filamentous phage-based systems have been used.

Initially proposed as display of single-chain Fv (scFv) fragments (WO 88/06630; see additionally WO 92/01047), the method has rapidly been expanded to the display of bovine pancreatic trypsin inhibitor (BPTI) (WO 90/02809), peptide libraries (WO 91/19818), human growth hormone (WO 92/09690), and of various other proteins including the display of multimeric proteins such as Fab fragments (WO 91/17271; WO 92/01047).

To anchor the peptide or protein to the filamentous bacteriophage surface, mostly genetic fusions to phage coat proteins are employed. Preferred are fusions to gene III protein (Parmley & Smith, 1988) or fragments thereof (Bass et al., 1990), and gene VIII protein (Greenwood et al., 1991). In one case, gene VI has been used (Jespers et al., 1995), and recently, a

combination of gene VII and gene IX has been used for the display of Fv fragments (Gao et al., 1999).
Furthermore, phage display has also been achieved on phage lambda. In that case, gene V protein (Maruyama et al., 1994), gene J protein, and gene D protein (Sternberg & Hoess, 1995; Mikawa et al., 1996) have been used.

Besides using genetic fusions, foreign peptides or proteins have been attached to phage surfaces via association domains. In WO 91/17271, it was suggested to use a tag displayed on phage and a tag binding ligand fused to the peptide/protein to be displayed to achieve a non-covalent display.
A similar concept was pursued for the display of cDNA libraries (Crameri & Suter, 1993). There the jun/fos interaction was used to mediate the display of cDNA fragments. In their construct, additional cysteine residues flanking both ends of jun as well as fos further stabilised the interaction by forming two disulfide bonds.

When screening phage display libraries in biopanning the problem remains how best to recover phage which have bound to the desired target. Normally, this is achieved by elution with appropriate buffers, either by using a pH- or salt gradient, or by specific elution using soluble target. However, the most interesting binders which bind with high affinity to the target might be lost by that approach. Several alternative methods have been devised which try to overcome that problem, either by providing a cleavage signal between the (poly)peptide/protein being displayed and its fusion partner, or between the target of interest and its carrier which anchors the target to a solid surface.

Furthermore, all the approaches referred to hereinabove require to use fusion proteins comprising at least part of a phage coat protein and a foreign (poly)peptide/protein. Especially in the case of using gene III as partner for peptides/proteins to be displayed, this leads to several problems. First, the expression product of gene III is toxic to the host cell, which requires tight regulation of gene III fusion proteins. Second, expression of gene III products can make host cells resistant to infection with helper phage required for the production of progeny phage particles. And finally, recombination events between gene III fusion constructs and wild type copies of gene III lead to undesired artefacts. Furthermore, since at least the C-terminal domain of the gene III protein comprising about 190 amino acids has to be used in

order to achieve incorporation of the fusion protein into the phage coat, the size of the vectors comprising the nucleic acid sequences is rather larger, leading to a decrease in transformation efficiency. Transformation efficiency, however, is a crucial factor for the production of very large libraries. Additionally, for the characterisation of (poly)peptide/proteins obtained after selection from a phage display library, the (poly)peptide/protein are usually recloned into expression vectors in order to remove the phage coat protein fusion partner, or in order to create new fusion proteins such as by fusion to enzymes for detection or to multimerisation domains. It would be advantageously to have a system which would allow direct expression without recloning, and direct coupling of the (poly)peptide/protein to other moieties.

Furthermore, most of these approaches (except for the work of Jespers et al. (1995), WO 91/17271, and Crameri & Suter (1993) mentioned hereinabove) are limited to the presentation of (poly)peptides/proteins having a free N-terminus, since the (poly)peptides/proteins have to be fused at the C-terminus with a phage coat protein. Especially in the case of cDNA libraries, or in the case of proteins requiring a free C-terminus to be functional, it would be highly desirable to have a simple method which doesn't require the generation of C-terminal fusions.

SUMMARY OF THE INVENTION

Thus, the technical problem underlying the present invention is to develop a simple, reliable system which enables the presentation of (poly)peptides/proteins on phage particles without the need to use fusion proteins with phage coat proteins. Additionally, there is a need for a method which allows to recover tightly binding (poly)peptides/proteins in a more reliable way.

The solution to this technical problem is achieved by providing the embodiments characterised in the claims. Accordingly, the present invention allows to easily create and screen large libraries of (poly)peptides/proteins displayed on the surface of bacteriophage particles. The technical approach of the present invention, i.e. linking (poly)peptides/proteins by disulfide bonds to the surface of phage particles, is neither provided nor suggested by the prior art.

Thus, the present invention relates to a method for displaying a (poly)peptide/protein on the surface of a bacteriophage particle comprising:

causing or allowing the attachment of said (poly)peptide/protein after expression to a member of the protein coat of said bacteriophage particle, wherein said attachment is caused by the formation of a disulfide bond between a first cysteine residue comprised in said (poly)peptide/protein and a second cysteine residue comprised in said member of the protein coat.

DETAILED DESCRIPTION OF THE INVENTION

In the context of the present invention, the term "bacteriophage" relates to bacterial viruses forming packages consisting of a protein coat containing nucleic acid required for the replication of the phages. The nucleic acid may be DNA or RNA, either double or single stranded, linear or circular. Bacteriophage such as phage lambda or filamentous phage (such as M13, fd, or f1) are well known to the artisan of ordinary skill in the art. In the context of the present invention, the term "bacteriophage particles" refers to the particles according to the present invention, i.e. to particles displaying a (poly)peptide/protein via a disulfide bonds.

During the assembly of bacteriophages, the coat proteins may package different nucleic acid sequences, provided that they comprise a packaging signal. In the context of the present invention, the term "nucleic acid sequences" contained in bacteriophages or bacteriophage particles relates to nucleic acid sequences or vectors having the ability to be packaged by bacteriophage coat proteins during assembly of bacteriophages or bacteriophage particles. Preferably said nucleic acid sequences or vectors are derived from naturally occurring genomes of bacteriophage, and comprise for example, in the case of filamentous phage, phage and phagemid vectors. The latter are plasmids containing a packaging signal and a phage origin of replication in addition to plasmid features.

The term "(poly)peptide" relates to molecules consisting of one or more chains of multiple, i. e. two or more, amino acids linked via peptide bonds.

The term "protein" refers to (poly)peptides where at least part of the (poly)peptide has or is able to acquire a defined three-dimensional arrangement by forming secondary, tertiary, or quaternary structures within and/or between its (poly)peptide chain(s). This definition comprises proteins such as naturally occurring or at least partially artificial proteins, as well as fragments or domains of whole proteins, as long as these fragments or domains are able to acquire a defined three-dimensional arrangement as described above.

Examples of (poly)peptides/proteins consisting of one chain are single-chain Fv antibody fragments, and examples for (poly)peptides/proteins consisting of more chains are Fab antibody fragments.

When the first cysteine residue is located at the C-terminus of the (poly)peptide/protein, the display format corresponds to the conventional display set-up with the C-terminus being genetically fused to the member of the phage coat protein. However, by using the N-terminus of the (poly)peptide/protein, the display format can be reverted as in the pJuFO system of Crameri & Suter referred to above.

The term "surface of a bacteriophage particle" refers to the part of a bacteriophage particle which is in contact with the medium the particle is contained in and which is accessible. The surface is determined by the proteins being part of the phage coat (the members of the protein coat of the particle) which is assembled during phage production in appropriate host cells.

The term "after expression" refers to the situation that nucleic acid encoding said (poly)peptide/protein is expressed in a host cell prior to attachment of the (poly)peptide/protein to said coat, in contrast to approaches where nucleic acid encoding fusion proteins with bacteriophage coat proteins are being expressed. The expression of nucleic acid encoding said (poly)peptide/protein and the step of causing or allowing the attachment may be performed in separated steps and/or environments. Preferably, however, expression and the step of causing or allowing the attachment are being performed sequentially in an appropriate host cell. The term "wherein said attachment is caused by the formation of a disulfide bond" refers to a situation, wherein the disulfide bond is responsible for the attachment, and wherein no interaction domain for interaction with a second domain present in the (poly)peptide/protein has been recombinantly fused to said member of the protein coat, as for example in the case of the pJuFo system (Crameri & Suter, 1993).

In a preferred embodiment, the bacteriophage particle displaying the (poly)peptide/protein contains a nucleic acid sequence encoding the (poly)peptide/protein.

Methods for construction of nucleic acid molecules encoding a (poly)peptide/protein according to the present invention, for construction of vectors comprising said nucleic acid molecules, for introduction of said vectors into appropriately chosen host cells, for causing or allowing the expression of said (poly)peptides/proteins are well-known in the art (see, e.g., Sambrook et al., 1989; Ausubel et al., 1999; Ge et al, 1995). Further well-known are methods

for the introduction of genetic material required for the generation of progeny bacteriophages or bacteriophage particles in appropriate host cells, and for causing or allowing the generation of said progeny bacteriophages or bacteriophage particles (see, e.g., Kay et al., 1996).

In a further preferred embodiment, the present invention relates to a method, wherein said second cysteine residue is present at a corresponding amino acid position in a wild type coat protein of a bacteriophage.

In a yet further preferred embodiment, the present invention relates to a method, wherein said member of the protein coat is a wild type coat protein of a bacteriophage.

The term "wild type coat protein" refers to those proteins forming the phage coat of naturally occurring bacteriophages. In the case of filamentous bacteriophage, said wild type proteins are gene III protein (pIII), gene VI protein (pVI), gene VII protein (pVII), gene VIII protein (pVIII), and gene IX protein (pIX). The sequences, including the differences between the closely related members of the filamentous bacteriophages such as f1, fd, and M13, are well known to one of ordinary skill in the art (see, e.g., Kay et al., 1996).

In a further preferred embodiment, said member of the protein coat is a truncated variant of a wild type coat protein of a bacteriophage, wherein said truncated variant comprises at least that part of said wild type coat protein causing the incorporation of said coat protein into the protein coat of the bacteriophage particle.

The term "truncated variant" refers to proteins derived from the wild type proteins referred to above which are modified by deletion of at least part of the wild type sequences. This comprises variants such as truncated gene III protein variants which have been found in bacteriophage mutants (Crissman & Smith, 1984) or which have been generated in the course of standard phage display methods (e.g. Bass et al., 1990; Krebber, 1996). For example, said truncated variant may consist, or include, the C-terminal domain of the gene III protein. To identify truncated variants according to the present invention, a detection tag may be fused to the variant, and an assay may be set up to determine whether the variant is incorporated into the phage coat of bacteriophage particles formed in the presence of the variant.

By way of truncating a wild type protein by deleting a part of the wild type protein, a cysteine residue may become available which in the wild type protein was forming a disulfide bond with a second cysteine comprised in the deleted part.

In a yet further preferred embodiment, said member of the protein coat is a modified variant of a wild type coat protein of a bacteriophage, wherein said modified variant is capable of being incorporated into the protein coat of the bacteriophage particle.

Methods for achieving modification of a wild type protein according to the present invention are well-known to one of ordinary skill in the art, and involve standard cloning and/or mutagenesis techniques. Methods for the construction of nucleic acid molecules encoding a modified variant of a wild type protein used in a method according to the present invention, for construction of vectors comprising said nucleic acid molecules, including the construction of phage and/or phagemid vectors, for introduction of said vectors into appropriately chosen host cells, for causing or allowing the expression of said modified protein are well-known in the art (see, e.g., Sambrook et al., 1989; Ausubel et al., 1999; Kay et al., 1996). To identify modified variants according to the present invention, a detection tag may be fused to the variant, and an assay may be set up to determine whether the variant is capable or being incorporated into the phage coat of bacteriophage particles formed in the presence of the variant.

In a most preferred embodiment, said second cysteine residue is not present at a corresponding amino acid position in a wild type coat protein of a bacteriophage.

In a preferred embodiment, said second cysteine has been artificially introduced into a wild type coat protein of a bacteriophage.
In the context of the present invention, the term "artificially introduced" refers to a situation where a wild type coat protein has been modified by e.g. recombinant means. For example, nucleic acid encoding a wild type coat protein may be manipulated by standard procedures to introduce a cysteine codon creating a nucleic acid sequence encoding a modified coat protein, wherein a cysteine residue is artificially introduced by insertion into, or addition of said cysteine residue to, said at least part of a wild type or modified coat protein, or by substitution of an amino acid residue comprised in said at least part of a wild type or modified protein by

said cysteine residue, or by fusion of said at least part of a wild type or modified coat protein with a (poly)peptide/protein comprising said second cysteine residue, or by any combination of said insertions, additions, substitutions or fusions. Upon expression of the nucleic acid comprising such recombinantly introduced cysteine codon, a variant of the wild type protein is formed comprising a cysteine residue.

In a further most preferred embodiment, said second cysteine has been artificially introduced into a truncated variant of a wild type coat protein of a bacteriophage.

In a yet further preferred embodiment, said second cysteine has been artificially introduced into a modified variant of a wild type coat protein of a bacteriophage.

Methods for achieving the artificial introduction according to the present invention are well-known to one of ordinary skill in the art, and involve standard cloning and/or mutagenesis techniques. Methods for the construction of nucleic acid molecules encoding a modified variant of a wild type protein used in a method according to the present invention, for construction of vectors comprising said nucleic acid molecules, for introduction of said vectors into appropriately chosen host cells, for causing or achieving the expression of said fusion proteins are well-known in the art (see, e.g., Sambrook et al., 1989; Ausubel et al., 1999).

In another embodiment, the present invention relates to a method, wherein said second cysteine is present at, or in the vicinity of, the C- or the N-terminus of said member of the phage coat of said bacteriophage particle.

The term "in the vicinity of" refers to a stretch of up to 15, or more preferably, up to 10 amino acids, counted in both cases from either N- or C-terminus of said (poly)peptide/protein, provided that the N- or C-terminus is located at the outside of the bacteriophage.

Yet further preferred is a method, wherein said bacteriophage is a filamentous bacteriophage. Filamentous bacteriophage such as M13, fd, or f1 are well known to the artisan of ordinary skill in the art.

In the case of filamentous bacteriophage, a method is particularly preferred, wherein said member of the protein coat of the bacteriophage particle is or is derived from the wild type coat protein pIII.
Further preferred is a method, wherein said member of the protein coat of the bacteriophage particle is or is derived from the wild type coat protein pIX.
In the context of the present invention, the term "is derived" refers to a modification, wherein the modified protein is capable of being incorporated into the protein coat of the bacteriophage particle. Preferably, those parts of the modified protein corresponding to the wild type protein exhibit an amino acid identity exceeding about 70%, preferably about 80%, most preferably about 90% compared to the corresponding wild type sequence.

In a yet further preferred embodiment of the present invention, the method comprises:
(a) providing a host cell harbouring a nucleic acid sequence comprising a nucleic acid sequence encoding said (poly)peptide/protein;
(b) causing or allowing the expression of said nucleic acid sequence; and
(c) causing or allowing the production of bacteriophage particles in said host cell.

In the context of the present invention, the term "causing or allowing the expression" describes cultivating host cells under conditions such that nucleic acid sequence is expressed.
Methods for construction of nucleic acid molecules encoding a (poly)peptide/protein according to the present invention, for construction of vectors comprising said nucleic acid molecules, for introduction of said vectors into appropriately chosen host cells, for causing or allowing the expression of (poly)peptides/proteins are well-known in the art (see, e.g., Sambrook et al., 1989; Ausubel et al., 1999). Further well-known are methods for the introduction of genetic material required for the generation of progeny bacteriophages or bacteriophage particles in appropriate host cells, and for causing or allowing the generation of said progeny bacteriophages or bacteriophage particles (see, e.g., Kay et al., 1996). The step of causing or allowing the production of bacteriophage particles may require the use of appropriate helper phages, e.g. in the case of working with phagemids.
The steps (b) and (c) may be performed sequentially, in either order, or simultaneously.

In a still further embodiment, said (poly)peptide/protein comprises an immunoglobulin or a functional fragment thereof.

In this context, "immunoglobulin" is used as a synonym for "antibody". The term "functional fragment" refers to a fragment of an immunoglobulin which retains the antigen-binding moiety of an immunoglobulin. Functional immunoglobulin fragments according to the present invention may be Fv (Skerra & Plückthun, 1988), scFv (Bird et al., 1988; Huston et al., 1988), disulfide-linked Fv (Glockshuber et al., 1992; Brinkmann et al., 1993), Fab, $F(ab')_2$ fragments or other fragments well-known to the practitioner skilled in the art, which comprise the variable domain of an immunoglobulin or immunoglobulin fragment.
Particularly preferred is an scFv or Fab fragment.

In a preferred embodiment, the present invention relates to a nucleic acid sequence encoding a modified variant of a wild type coat protein of a bacteriophage, wherein said modified variant consists of:
(a) one or more parts of said wild type coat protein of a bacteriophage, wherein one of said parts comprises at least that part which causes or allows the incorporation of said coat protein into the phage coat; and
(b) between one and six additional amino acid residues not present at the corresponding amino acid positions in a wild type coat protein of a bacteriophage, wherein one of said additional amino acid residues is a cysteine residue.

In the context of the present invention, a modified variant obtained by substitution of an amino acid residue in a wild type coat protein sequence by a cysteine residue may be regarded as a variant composed of two parts of said wild type protein linked by an additional cysteine residue. Correspondingly, variants of a wild type coat protein comprising several mutations compared to the wild type sequence may be regarded as being composed of several wild type parts, wherein the individual parts are linked by the mutated residues. However, said variant may also result from the addition of up to six residues, including a cysteine residue, to either C- and or N-terminus of the wild type coat protein.

Further preferred is a nucleic acid sequence encoding a modified variant of a wild type coat protein of a bacteriophage, wherein said modified variant consists of:

(a) one or more parts of said wild type coat protein of a bacteriophage, wherein one of said parts comprises at least that part which causes or allows the incorporation of said coat protein into the phage coat;
(b) between one and six additional amino acid residues not present at the corresponding amino acid positions in a wild type coat protein of a bacteriophage, wherein one of said additional amino acid residues is a cysteine residue; and
(c) one or more peptide sequences for purification and/or detection purposes.

Particularly preferred are peptides comprising at least five histidine residues (Hochuli et al., 1988), which are able to bind to metal ions, and can therefore be used for the purification of the protein to which they are fused (Lindner et al., 1992). Also provided for by the invention are additional moieties such as the commonly used c-myc and FLAG tags (Hopp et al., 1988; Knappik & Plückthun, 1994), or the Strep-tag (Schmidt & Skerra, 1994; Schmidt et al., 1996).

The modified variant may further comprise amino acid residues required for cloning, for expression, or protein transport. Amino acid residues required for cloning may include residues encoded by nucleic acid sequences comprising recognition sequences for restriction endonucleases which are incorporated in order to enable the cloning of the nucleic acid sequences into appropriate vectors. Amino acid residues required for expression may include residues leading to increased solubility or stability of the (poly)peptide/protein. Amino acid residues required for protein transport may include signalling sequences responsible for the transport of the modified variant to the periplasm of *E.coli*, and/or amino acid residues facilitating the efficient cleavage of said signalling sequences. Further amino acid residues required for cloning, expression, protein transport, purification and/or detection purposes referred to above are numerous moieties well known to the practitioner skilled in the art.

In another embodiment, the present invention relates to a vector comprising a nucleic acid sequence according to the present invention.

In a preferred embodiment, the vector further comprises one or more nucleic acid sequences encoding a (poly)peptide/protein comprising a second cysteine residue.

In a most preferred embodiment, said (poly)peptide/protein comprises an immunoglobulin or a functional fragment thereof.
In the case of single-chain Fv antibody fragments referred to hereinabove, the vector comprises one nucleic acid sequence encoding the VH and VL domains linked by a (poly)peptide linker, and in the case of Fab antibody fragments, the vector comprises two nucleic acid sequences encoding the VH-CH and the VL-CL chains.

In a further embodiment, the present invention relates to a host cell containing a nucleic acid sequence according to the present invention or a vector according to the present invention.

In the context of the present invention the term "host cell" may be any of a number commonly used in the production of heterologous proteins, including but not limited to bacteria, such as *Escherichia coli* (Ge et al., 1995), or *Bacillus subtilis* (Wu et al., 1993), fungi, such as yeasts (Horwitz et al., 1988; Ridder et al., 1995) or filamentous fungus (Nyyssönen et al., 1993), plant cells (Hiatt & Ma, 1993; Whitelam et al., 1994), insect cells (Potter et al., 1993; Ward et al., 1995), or mammalian cells (Trill et al., 1995).

In a yet further preferred embodiment, the present invention relates to a modified variant of a wild type bacteriophage coat protein encoded by a nucleic acid sequence according to the present invention, a vector according to the present invention or produced by a host cell according to the present invention.

In another embodiment, the present invention relates to a bacteriophage particle displaying a (poly)peptide/protein on its surface obtainable by a method comprising:
causing or allowing the attachment of said (poly)peptide/protein after expression to a member of the protein coat of said bacteriophage particle, wherein said attachment is caused by the formation of a disulfide bond between a first cysteine residue comprised in said (poly)peptide/protein and a second cysteine residue comprised in said member of the protein coat.

In another embodiment, the present invention relates to a bacteriophage particle displaying a (poly)peptide/protein attached to its surface, wherein said attachment is caused by the formation of a disulfide bond between a first cysteine residue comprised in said

(poly)peptide/protein and a second cysteine residue comprised in a member of the protein coat of said bacteriophage particle.

In a preferred embodiment, the bacteriophage particle further contains a vector comprising one or more nucleic acid sequences encoding said (poly)peptide/protein.

In a most preferred embodiment of the present invention, the bacteriophage particle contains a vector according to the present invention, wherein said vector comprises a nucleic acid sequence encoding a modified wild type bacteriophage coat protein and furthermore one or more nucleic acid sequences encoding a (poly)peptide/protein and most preferably comprising at least a functional domain of an immunoglobulin.

The preferred embodiments of the method of the present invention referred to hereinabove mutatis mutandis apply to the bacteriophages of the present invention.

In a further embodiment, the present invention relates to a diverse collection of bacteriophage particles according to the present invention, wherein each of said bacteriophage particles displays a (poly)peptide/protein out of a diverse collection of (poly)peptides/proteins.
A "diverse collection of bacteriophage particles" may as well be referred to as a "library" or a "plurality of bacteriophage particles". Each member of such a library displays a distinct member of the library.

In the context of the present invention the term "diverse collection" refers to a collection of at least two particles or molecules which differ in at least part of their compositions, properties, and/or sequences. For example, a diverse collection of (poly)peptides/proteins is a set of (poly)peptides/proteins which differ in at least one amino acid position of their sequence. Such a diverse collection of (poly)peptides/proteins can be obtained in a variety of ways, for example by random mutagenesis of at least one codon of a nucleic acid sequence encoding a starting (poly)peptide/protein, by using error-prone PCR to amplify a nucleic acid sequence encoding a starting (poly)peptide/protein, or by using mutator strains as host cells in a method according to the present invention. These and additional or alternative methods for the generation of diverse collections of (poly)peptides/proteins are well-known to one of ordinary skill in the art. A "diverse collection of bacteriophage particles" may be referred to as a library

or a plurality of bacteriophage particles. Each member of such a library displays a distinct member of the library.

In another embodiment, the invention relates to a method for obtaining a (poly)peptide/protein having a desired property comprising:
(a) providing the diverse collection of bacteriophage particles according to the present invention; and
(b) screening said diverse collection and/or selecting from said diverse collection to obtain at least one bacteriophage particle displaying a (poly)peptide/protein having said desired property.

In the context of the present invention the term "desired property" refers to a predetermined property which one of the (poly)peptides/proteins out of the diverse collection of (poly)peptides/proteins should have and which forms the basis for screening and/or selecting the diverse collection. Such properties comprise properties such as binding to a target, blocking of a target, activation of a target-mediated reaction, enzymatic activity, and further properties which are known to one of ordinary skill. Depending on the type of desired property, one of ordinary skill will be able to identify format and necessary steps for performing screening and/or selection.

Most preferred is a method , wherein said desired property is binding to a target of interest.

Said target of interest can be presented to said diverse collection of bacteriophage particles in a variety of ways well known to one of ordinary skill, such as coated on surfaces for solid phase biopanning, linked to particles such as magnetic beads for biopanning in solution, or displayed on the surface of cells for whole cell biopanning or biopanning on tissue sections. Bacteriophage particles having bound to said target can be recovered by a variety of methods well known to one of ordinary skill, such as by elution with appropriate buffers, either by using a pH- or salt gradient, or by specific elution using soluble target.

In a preferred embodiment, the method for obtaining a (poly)peptide/protein further comprises:
(ba) contacting said diverse collection of bacteriophage particles with the target of interest;

(bb) eluting bacteriophage particles not binding to the target of interest;

(bc) eluting bacteriophage particles binding to the target of interest by treating the complexes of target of interest and bacteriophages binding to said target of interest formed in step (ba) under reducing conditions.

Under reducing conditions, such as by incubation with DTT, the disulfide bonds are cleaved, thus allowing to recover the specific bacteriophage particles for further rounds of biopanning and/or for identification of the (poly)peptide/proteins specifically binding to said target.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure legends

Figure 1a: Vector map of construct pMorphX7-hag2-LH.

Figure 1b: Vector sequence of pMorphX7-hag2-LH.

Figure 2: Vector sequence of pTFT74-N1-hag-HIPM

Figure 3: Vector sequence of pQE60-MacI

Figure 4: Specific binding of scFv displayed on non-engineered phages.
Phages derived from constructs pMorphX7-MacI5-LCH, pMorphX7-MacI5-LHC and pMorphX7-MacI5-LH were produced by standard procedures and pre-incubated in PBSTM either with 5 mM DTT (+ DTT) or without DTT. 5 µg/well of specific antigen (MacI, dark columns) as well as unspecific control antigen (BSA, light columns) were coated onto Maxisorp Nunc-Immuno microtiter plates and incubated with 1x 10^{10} phages/well, respectively. Bound phages were detected via anti-M13-HRP conjugate and BM blue soluble substrate. Phages derived from conventional phage display vector pMorph13-MacI5 were used as control (3x 10^{7} phages/well). Experimental details are given in Example 1.

Figure 5: Specific binding of scFv displayed on non-engineered phages.
Phages derived from constructs pMorphX7-hag2-LCH, pMorphX7-hag2-LHC and pMorphX7-hag2-LH were produced by standard procedures and pre-incubated in PBSTM either with 5 mM DTT (+ DTT) or without DTT. 5 µg/well of specific antigen (N1-hag, dark columns) as well as unspecific control antigen (BSA, light columns) were coated onto Maxisorp Nunc-Immuno microtiter plates and incubated with 1x 10^{10} phages/well, respectively. Bound phages were detected via anti-M13-HRP conjugate and BM blue soluble substrate. Phages derived from conventional phage display vector pMorph13-hag2 were used as control (3x 10^{7} phages/well). Experimental details are given in Example 1.

Figure 6a: Vector map of construct pBR-C-gIII.

Figure 6b: Sequence of expression cassette for full length pIII with an N-terminal cysteine residue (C-gIII).

Figure 6c: Sequence of expression cassette for truncated pIII with an N-terminal cysteine residue (C-gIIICT).

Figure 7a: Vector map of construct pMorph18-C-gIII- hag2-LHC.

Figure 7b: Vector sequence of pMorph18-C-gIII-hag2-LHC.

Figure 8: Detection of scFv MacI-5 displayed on engineered phages – Two-vector system. Phages derived from constructs pMorphX7-MacI-5-LH / pBR-C-gIII (lanes 1 & 5), pMorphX7-MacI-5-LHC / pBR-C-gIII (lanes 2 & 6), pMorphX7-MacI-5-LHC (lanes 3 & 7) and pMorphX7-MacI-5-LH (lanes 4 & 8) were produced by standard procedures. 1 – 5 x 10^{10} phages were pre-incubated in PBS with DTT (lanes 1 – 4) or without DTT (lanes 5 – 8). SDS loading buffer lacking reducing agents was added, phages were applied to an 4-15% SDS PAA Ready gel and analysed in immunoblots. Detection of scFvs associated with phages was done via anti-FLAG M1 antibody, anti-mouse-IgG-AP conjugate and Fast BCIP/NPT substrate (6A) and via anti-pIII antibody, anti-mouse-IgG-AP conjugate and Fast BCIP/NPT substrate (6B). Low range marker (Amersham #RPN756) is marked as M. Experimental details are given in Example 2.1.

Figure 9: Detection of scFvs displayed on engineered phages – One-vector system. Phages derived from constructs pMorph18-C-gIII-hag2-LHC (lanes 1 – 8; 7A), pMorph18-C-gIII-AB1.1-LHC (lanes 1, 2, 5 and 6; 7B) and pMorph18-C-gIII-MacI-5-LHC (lanes 3, 4, 7 and 8; 7B) were produced by standard procedures. 1-5 x 10^{10} phages were pre-incubated in PBS with DTT (lanes 1, 2, 5 and 6; 7A and lanes 1 – 4; 7B) or without DTT (lanes 3, 4, 7 and 8; 7A and lanes 5 – 8; 7B). SDS loading buffer lacking reducing agents was added, phages were applied to an 4-15% SDS PAA Ready gel and analysed in immunoblots. Detection of scFvs associated with phages was done via anti-FLAG M1 antibody, anti-mouse-IgG-AP conjugate and Fast BCIP/NPT substrate (lanes 1 – 4; 7A) and via anti-pIII antibody, anti-mouse-IgG-AP conjugate and Fast BCIP/NPT substrate (lanes 5 – 8; 7A and lanes 1 –8; 7B).

Low range marker (Amersham #RPN756) is marked as M. Experimental details are given in Example 2.1.

Figure 10: Specific binding of scFv displayed on engineered phages – Comparison of the different two-vector systems.

Phages derived from constructs pMorphX7-MacI-5-LHC / pBR-C-gIII (1), pMorphX7-MacI-5-LHC / pBR-C-gIIICT (2), pMorphX7-MacI-5-LHC / pUC-C-gIII (3), pMorphX7-MacI-5-LHC / pUC-C-gIIICT (4), pMorphX7-MacI-5-LHC (5), pMorphX7-MacI-5-LH (6) and the conventional phage display vector pMorph13-MacI-5 (7) were produced by standard procedures. 5 µg of specific antigen (MacI) as well as unspecific control antigen (BSA, data not shown) were coated onto Maxisorp Nunc-Immuno microtiter plates and incubated with a range of 6.4×10^6 and 1×10^{11} phages per well. Bound phages were detected via anti-M13-HRP conjugate and BM blue substrate. Experimental details are given in Example 2.1.

Figure 11: Specific binding of scFv displayed on engineered phages – Comparison of the one- and two-vector system.

Phages derived from constructs pMorphX7-MacI-5-LHC / pBR-C-gIII (1), pMorphX7-MacI-5-LHC / pBR-C-gIIICT (2), pMorph18-C-gIII-MacI-5-LHC (3), pMorph18-C-gIIICT-MacI-5-LHC (4) and pMorphX7-MacI-5-LHC (5) were produced by standard procedures. 5 µg of specific antigen (MacI, dark columns) as well as unspecific control antigen (BSA, light columns) were coated onto Maxisorp Nunc-Immuno microtiter plates and incubated with $1x\ 10^{10}$ and $1x\ 10^9$ phages, respectively. Bound phages were detected via anti-M13-HRP conjugate and BM blue substrate. Experimental details are given in Example 2.1.

Figure 12: Specific binding of scFv displayed on engineered phages – Comparison of engineered gene III and gene IX proteins in the one-vector system.

Phages derived from constructs pMorph18-C-gIII-MacI-5-LHC (1), pMorph18-C-gIIICT-MacI-5-LHC (2), pMorph18-C-gIX-MacI-5-LHC (3), pMorphX7-MacI-5-LHC (4) and the conventional phage display vector pMorph13-MacI-5 (5) were produced by standard procedures. 5 µg of specific antigen (MacI, dark columns) as well as unspecific control antigen (BSA, light columns) were coated onto Maxisorp Nunc-Immuno microtiter plates and incubated with $1x\ 10^{10}$, $1x\ 10^9$ and $1x\ 10^8$ phages, respectively. Bound phages were detected

via anti-M13-HRP conjugate and BM blue substrate. Experimental details are given in Example 2.1.

Figure 13: Specific binding of scFv displayed on engineered phages – Impact of DTT. Phages derived from constructs pMorph18-C-gIII-MacI-5-LHC (1), pMorph18-C-gIIICT-MacI-5-LHC (2), pMorph18-C-gIX-MacI-5-LHC (3), pMorphX7-MacI-5-LHC (4) and the conventional phage display vector pMorph13-MacI-5 (5) were produced by standard procedures and pre-incubated in PBSTM either with 5 mM DTT (+) or without DTT (-). 5 µg of specific antigen (MacI, dark columns) as well as unspecific control antigen (BSA, light columns) were coated onto Maxisorp Nunc-Immuno microtiter plates and incubated with 1x 10^{10} phages respectively. Bound phages were detected via anti-M13-HRP conjugate and BM blue substrate. Experimental details are given in Example 2.1.

Figure 14: Specificity of selected scFvs – Panning of pre-selected pools against N1-MacI. scFvs selected after two rounds of cys-display panning against antigen N1-MacI from the κ-chain (1-5) and the λ-chain pool (6-8) were expressed according to standard procedures. 0.1 µg/well of milk powder (A), BSA (B), FITC-BSA (C, FITC coupled to BSA), N1-hag (D), N1-Np50 (E) and N1-MacI (N1-MacI) was coated onto 384 well plates (Maxisorp; Nunc) and incubated with 10 µl scFv solution, respectively. Bound scFvs were detected via a mixture of anti-Flag M1, anti-Flag M2 and anti-mouse IgG-AP conjugate as well as AttoPhos fluorescence substrate (Roche #1484281). Each scFv was tested in quadruplicates and mean values are presented.

Figure 15: Specificity of selected scFvs – Panning of pre-selected pools against N1-Np50. scFvs selected after two rounds of cys-display panning against antigen N1-Np50 (1-8) were expressed according to standard procedures. 0.1 µg/well of milk powder (A), BSA (B), FITC-BSA (C, FITC coupled to BSA), N1-hag (D), N1-MacI (E) and N1-Np50 (N1-Np50) was coated onto 384 well plates (Maxisorp; Nunc) and incubated with 10 µl scFv solution, respectively. Bound scFvs were detected via a mixture of anti-Flag M1, anti-Flag M2 and anti-mouse IgG-AP conjugate as well as AttoPhos fluorescence substrate (Roche #1484281). Each scFv was tested in quadruplicates and mean values are presented.

Figure 16a: Vector map of construct pMorphX10-Fab-MacI5-VL-LHC-VH-FS.

Figure 16b: Complete vector sequence of pMorphX10-Fab-MacI5-VL-LHC-VH-FS.

Figure 17: Detection of Fab ICAM1-C8 displayed on engineered phages.
Phages derived from constructs pMorphX10-Fab-ICAM1C8-VL-LHC-VH-MS / pBAD-SS-C-gIII (lanes 5,6,11,12), pMorphX10-Fab-ICAM1C8-VL-LHC-VH-MS (lanes 3,4,9,10) and pMorph18-Fab-ICAM1C8 (lanes 1,2,7,8) were produced by standard procedures. 1 x 10^{10} phages were pre-incubated in PBS with DTT (lanes 1 – 6) or without DTT (lanes 7 – 12). SDS loading buffer lacking reducing agents was added, phages were applied to an 12 % SDS PAA Ready gel and analysed in immunoblots. Detection was done via anti-pIII antibody, anti-mouse-IgG-HRP conjugate and BM Blue POD precipitating substrate. Low range molecular weight marker (Amersham Life Science #RPN756) is marked as M. Experimental details are given in Example 2.2.

Figure 18: Detection of Fab MacI-A8 displayed on engineered phages.
Phages derived from constructs pMorphX10-Fab-MacIA8-VL-LHC-VH-FS / pBAD-SS-C-gIII (lanes 5,6,11,12), pMorphX10-Fab-MacIA8-VL-LHC-VH-FS (lanes 3,4,9,10) and pMorph18-Fab-MacIA8 (lanes 1,2,7,8) were produced by standard procedures. 1 x 10^{10} phages were pre-incubated in PBS with DTT (lanes 1 – 6) or without DTT (lanes 7 – 12). SDS loading buffer lacking reducing agents was added, phages were applied to an 12 % SDS PAA Ready gel and analysed in immunoblots. Detection was done via anti-pIII antibody, anti-mouse-IgG-HRP conjugate and BM Blue precipitating substrate. Low range molecular weight marker (Amersham Life Science #RPN756) is marked as M. Experimental details are given in Example 2.2.

Figure 19: Specific binding of Fabs displayed on engineered phages – Fab MacI-5.
Phages derived from constructs pMorphX10-Fab-MacI5-VL-LHC-VH-FS / pBR-C-gIII (1), pMorphX10-Fab-MacI5-VL-C-VH-FS / pBR-C-gIII (2), pMorphX10-Fab-MacI5-VL-VH-CFS / pBR-C-gIII (3), pMorphX10-Fab-MacI5-VL-VH-LHC / pBR-C-gIII (4), pMorphX9-Fab-MacI5-FS (5), and the conventional phage display vector pMorph18-Fab-MacI5 (6) were produced by standard procedures. 5 µg/well of specific antigen (N1-MacI) were coated onto Maxisorp Nunc-Immuno microtiter plates and incubated with 1x 10^{8} (light columns) and 1x 10^{9} (dark columns) phages per well. Bound phages were detected via anti-M13-HRP conjugate and BM blue soluble substrate. Each column represents the mean value of three

independent phage preparations tested in duplicates. Experimental details are given in Example 2.2.

Figure 20: Specific binding of Fabs displayed on engineered phages – Fab MacI-A8.
Phages derived from constructs pMorphX10-Fab-MacIA8-VL-LHC-VH-FS / pBR-C-gIII (1), pMorphX10-Fab-MacIA8-VL-C-VH-FS / pBR-C-gIII (2), pMorphX10-Fab-MacIA8-VL-VH-CFS / pBR-C-gIII (3), pMorphX10-Fab-MacIA8-VL-VH-LHC / pBR-C-gIII (4), pMorphX9-Fab-MacIA8-FS (5), and the conventional phage display vector pMorph18-Fab-MacIA8 (6) were produced by standard procedures. 5 µg/well of specific antigen (N1-MacI) were coated onto Maxisorp Nunc-Immuno microtiter plates and incubated with 1x 10^9 (light columns) and 1x 10^{10} (dark columns) phages per well. Bound phages were detected via anti-M13-HRP conjugate and BM blue soluble substrate. Each column represents the mean value of three independent phage preparations tested in duplicates. Experimental details are given in Example 2.2.

Figure 21: Specific binding of Fabs displayed on engineered phages – Fab ICAM1-C8.
Phages derived from constructs pMorphX10-Fab-ICAM1C8-VL-LHC-VH-MS / pBR-C-gIII (1), pMorphX10-Fab-ICAM1C8-VL-C-VH-MS / pBR-C-gIII (2), pMorphX10-Fab-ICAM1C8-VL-VH-CMS / pBR-C-gIII (3), pMorphX10-Fab-ICAM1C8-VL-VH-LHC / pBR-C-gIII (4), pMorphX9-Fab-ICAM1C8-MS (5), pMorphX9-Fab-ICAM1C8-MS / pBR-C-gIII (6) were produced by standard procedures. 5 µg/well of specific antigen (ICAM1, dark columns) or unspecific antigen (BSA, light columns) were coated onto Maxisorp Nunc-Immuno microtiter plates and incubated with 1x 10^9 phages per well. Bound phages were detected via anti-M13-HRP conjugate and BM blue soluble substrate. Each column represents the mean value of one phage preparation tested in duplicates. Experimental details are given in Example 2.2.

Figure 22: Specific binding of Fabs displayed on engineered phages – Impact of DTT.
Phages derived from constructs pMorphX10-Fab-MacI5-VL-LHC-VH-FS / pBR-C-gIII (1), pMorphX10-Fab-MacI5-VL-C-VH-FS / pBR-C-gIII (2), pMorphX10-Fab-MacI5-VL-VH-CFS / pBR-C-gIII (3), pMorphX10-Fab-MacI5-VL-VH-LHC / pBR-C-gIII (4), pMorphX9-Fab-MacI5-FS (5), and the conventional phage display vector pMorph18-Fab-MacI5 (6) were produced by standard procedures and pre-incubated in PBSTM either with 10 mM DTT (+) or

without DTT (-). 5 μg/well of specific antigen (N1-MacI, dark columns) as well as unspecific control antigen (BSA, light columns) were coated onto Maxisorp Nunc-Immuno microtiter plates and incubated with 1x 10^9 phages respectively. Bound phages were detected via anti-M13-HRP conjugate and BM blue substrate. Each column represents the mean value of one phage preparation tested in duplicates. Experimental details are given in Example 2.2.

The examples illustrate the invention.

Example 1: Display of (poly)peptides/proteins on the surface of non-engineered filamentous bacteriophage particles via formation of disulfide bonds

In the following example, all molecular biology experiments are performed according to standard protocols (Ausubel et al., 1999).

Construction of vectors expressing scFvs

All vectors used are derivatives of the high copy phagemid pMorphX7-LH (Figure 1a+b), a derivative of the pCAL vector series (WO 97/08320; Knappik et al., 2000). The expression cassette comprises the phoA signal sequence, a minimal binding site for the monoclonal antibody (mab) anti-FLAG M1 (Sigma #F-3040) (Knappik and Plückthun, 1994), a single chain fragment (scFv), a short linker (PGGSG) and a 6x histidine tag (6His; Hochuli et al., 1988) (Figure 1a). pMorphX7-LCH and pMorphX7-LHC have been generated by inserting oligonucleotide cassettes coding for Cys-6His and 6His-Cys, respectively, between the unique *Asc*I and *Hind*III sites of pMorphX7-LH (Figure1a, Table 1). All vectors express soluble scFv not genetically fused to any phage coat protein. The conventional phage display vector pMorph13 which is based on the pCAL4 vector described in WO 97/08320 and expresses a fusion of an scFv to the C-terminal part of phage protein pIII was used as positive control. The scFvs have been exchanged between the respective vectors via the unique *Xba*I and *Eco*RI sites (c.f. Figure 1a).

Description of the scFv – antigen interactions

All scFvs derive from a human combinatorial antibody library (HuCAL; WO 97/08320; Knappik et al., 2000). The HuCAL VH and VL consensus genes (described in WO 97/08320), and the CDR3 sequences of the scFvs are given in Table 2. Clone hag2 was selected against a peptide from influenza virus hemagglutinine (aa 99-110 from hemagglutinine plus additional flanking aa (shown in italics, *CAG*PYDVPDYASLRS*HH*), and clone MacI-5 against a

fragment (MacI) of human CR-3 alpha chain (SWISS-PROT entry P11215, aa 149 – 353 of human CR-3 alpha fused to a C-terminal sequence containing a 6x histidine tag). The corresponding antigens for ELISA and doped library experiments were obtained as follows. The hag2 specific antigen N1-hag was produced using expression vector pTFT74-N1-hag-HIPM, a derivative of vector pTFT74 (Freund et al., 1993) (Fig. 2). N1-hag comprises aa 1-82 of mature gene III protein of phage M13 containing an additional methionine residue at the N-terminus (N1) fused to the amino acid sequence PYDVPDYASLRS*HHHHHH* (hag) comprising aa 99-110 from influenza virus hemagglutinine and a 6x histidine tag (in italics). Expression, purification and refolding of N1-hag was done as described (Krebber, 1996; Krebber et al., 1997). As antigen for MacI-5, a purified fragment (MacI) of human CR-3 alpha chain (SWISS-PROT entry P11215) fused to a C-terminal 6x histidine tag was used. In detail, the expression cassette encodes an N-terminal methionine, amino acids 149 – 353 of human CR-3 alpha and amino acids IEGRHHHHHH. This cassette is flanked by unique restriction sites *Bsp*HI and *Hin*dIII and can e.g. be introduced into the unique *Nco*I and *Hin*dIII sites of pQE-60 (QIAGEN GmbH, Hilden, Germany), yielding expression vector pQE60-MacI (Fig. 3). Expression and purification was performed using standard methods (The QIAexpressionist™ 3rd edition: A handbook for high-level expression and purification of 6xHis-tagged proteins (July 1998). QIAGEN GmbH, Hilden, Germany). Bovine serum albumin (BSA, Sigma #A7906) was used as negative control antigen.

Functionality of scFvs displayed on non-engineered phages

To demonstrate that the displayed scFvs are functional with respect to recognition of their specific antigens phage ELISAs were performed. The analysis was done for the two HuCAL scFvs hag2 and MacI-5. Three expression systems differing in the modules fused to the C-terminus of the scFv were analysed, namely pMorphX7-LH, pMorphX7-LHC and pMorphX7-LCH.

Phages were produced according to standard procedures using helper phage VCSM13 (Kay et al., 1996). Specific antigen or control antigen (BSA, Sigma #A7906) was coated for 12 h at 4°C at a concentration of 5 µg/well in PBS to Nunc Maxisorp microtiter plates (# 442404). Phages were pre-incubated in PBSTM (PBS containing 5% skimmed milk powder and 0.1%

Tween 20), either with or without 5 mM DTT, for 2 h at room temperature before they were applied to the ELISA well coated with antigen at a concentration of 1x 10^{10} phages per well except for pMorph13 which was used at a concentration of 3x 10^{7} phages per well. After binding for 1 h at RT, non-specifically bound phages were washed away with PBS containing 0.05% Tween 20 and bound phages were detected in ELISA using an anti-M13-HRP conjugate (Amersham Pharmacia Biotech #27-9421-01) and BM blue soluble (Boehringer Mannheim #1484281). Absorbance at 370 nm was measured. ELISA signals obtained with the specific antigen were compared to those with the control antigen. Specific binding of scFv displaying phages to antigen could be shown. As an example two of such ELISAs for scFvs hag2 and MacI-5 are presented in Figures 4 and 5, respectively. With phages derived from pMorphX7-LCH and pMorphX7-LHC signals between 1.9 and 5.8 times above background were achieved. When 5 mM DTT was added to the phages prior to antigen binding during the pre-incubation step, the ELISA signal was decreased to almost background levels while DTT had no major effect on the conventional display phages (pMorph13).

Enrichment of non-engineered phages displaying scFv

To prove that non-engineered phages displaying scFvs can be enriched on specific antigen a so called doped library experiment was performed. Specific phages were mixed with a high excess of unspecific phages and three rounds of panning on specific antigen were performed. The enrichment for specific phages was determined after each round. The analysis was done for the two HuCAL scFvs hag2 and MacI-5 in the pMorphX7-LHC vector.

pMorphX7-hag2-LHC and pMorphX7-MacI-5-LHC derived phages were mixed at ratios of 1:10^{5} (pMorphX7-hag2-LHC panning) as well as 10^{5}:1 (pMorphX7-MacI-5-LHC panning). Three rounds of panning were performed on the hag2 and MacI-5 specific antigen, respectively. Phages were prepared by standard procedure and pre-blocked by mixing 1:1 with PBSTM (PBS, 5% skimmed milk powder, 0.1% Tween20) and incubation for 2 h at RT. Wells of a Nunc Maxisorp microtiter plate (#442404) were coated with specific antigen N1-hag (as well as BSA) at a concentration of 5 µg/well in PBS overnight at 4°C, and subsequently blocked with 400 µl PBSM (PBS, 5% skimmed milk powder) for 2 h at RT. For the first round, 10^{11} pre-blocked phages were applied per well and incubated for 1 h at RT on

a microtiter plate shaker. Phage solution was removed and wells were washed 3 times with PBST (PBS, 0.05% Tween20) and 3 times with PBS. Bound phages were eluted with 100 mM triethylamine according to standard protocols and used for infection of TG1 cells. In addition, residual phages were eluted by direct infection of TG1 added to the wells. After each round of panning on specific antigen the ratio of specific to unspecific phages was determined by analysing at least 46 independent infected cells via PCR. The PCR was performed according to standard protocols using single colonies as source of template and oligonucleotides specific for VH CDR3 and VL CDR3 of each scFv as primers. After 3 rounds of panning, ~ 4% positive clones (4 out of 93 clones analysed) were obtained for the pMorphX7-hag2-LHC panning and ~ 90% positive clones (82 out of 91 clones analysed) were obtained for the pMorphX7-MacI-5-LHC panning.

Example 2: Display of (poly)peptides/proteins on the surface of engineered filamentous bacteriophage particles via formation of disulfide bonds

Example 2.1: Display of scFvs

Example 1 described above shows that functional scFvs can be displayed on non-engineered phages via disulfide bonds. This system can be further improved, e.g. via engineering an exposed cysteine on a phage coat protein. One candidate phage coat protein is protein III (pIII) which is composed of three domains N1, N2 and pIIICT. Possible sites for positioning an unpaired cysteine residue are the linker regions between the domains or the exposed N-terminus of the domain or the pIIICT in a truncated pIII version. A further example would be phage coat protein IX (pIX) where the cysteine could e.g. be linked to the N-terminus of the full length protein. In principle the cassettes for expression of such engineered proteins can be placed on the vector which is providing the scFv (one-vector system), or on a separate vector (two-vector system).

In the following we will describe experiments in which we engineered both a full length and a truncated pIII version as well as pIX. These proteins were co-expressed in the same bacterial cell together with the scFv, either from the same phagemid (pMorph18-C-gIII-scFv-LHC

derivatives; one-vector system) or from a separate plasmid (pBR322-C-gIII or pUC19-C-gIII and derivatives; two-vector system).

Construction of vectors expressing scFvs and engineered phage coat proteins

Phage coat protein expression cassettes for the two-vector system were constructed as follows: Two different expression cassettes flanked by unique *Nhe*I and *Hin*dIII restriction sites at the ends were made positioning an unpaired cysteine residue at the exposed N-terminus of the N1-domain of full length mature pIII (C-gIII) or at the N-terminus of the pIIICT domain of the truncated protein (amino acids 216 to 406 of protein pIII; C-gIIICT) (Fig. 6b+c)). Both expression cassettes are under the control of the lac promotor/operator region and comprise the signal sequence ompA, amino acids DYCDIEF and the pIII or pIIICT ORF (complete amino acid sequences are given in Table 3). Plasmids expressing the modified pIII proteins were obtained by inserting these *Nhe*I-*Hin*dIII cassettes into plasmid pBR322 and pUC19 via the unique *Nhe*I and *Hin*dIII or *Xba*I and *Hin*dIII sites, respectively. As an example, the vector map of pBR-C-gIII is depicted in Figure 6a. The resulting plasmids, pBR-C-gIII, pBR-C-gIIICT, pUC-C-gIII and pUC-C-gIIICT, were co-transformed with pMorphX7-LHC phagemids expressing the modified scFv (Example 1) into *E.coli* TG1 selecting for both antibiotic markers.

In the one-vector system both the modified phage coat proteins as well as the modified scFv were expressed from a dicistronic phagemid under control of the lac promotor/operator region. The first expression cassette comprises the signal sequence ompA, amino acids DYCDIEF and the ORF for the respective phage coat protein or part thereof. The unpaired cysteine residue was linked to the exposed N-terminus of the N1-domain of full length mature pIII (C-gIII), to the N-terminus of the truncated protein III (amino acids 216 to 406 of protein pIII; C-gIIICT) and to the N-terminus of protein IX (C-gIX), respectively (amino acid sequences are given in Table 4). The second expression cassette comprises the phoA signal sequence, the ORF of the respective scFv, a short linker (PGGSG), a 6x histidine tag (6His; Hochuli et al., 1988) and the single cysteine residue (see pMorphX7-LHC, Table 1). The complete vector sequence of pMorph18-C-gIII-hag2-LHC coding for modified full length pIII as well as modified scFv hag2 and the respective vector map are given in Figure 7a+b. The

different phage coat proteins can be exchanged via *Eco*RI and *Stu*I in a three fragment cloning procedure due to a second *Eco*RI site at the 3' end of the scFvs. The different engineered scFvs can be cloned via the unique *Mfe*I and *Hin*dIII sites. A derivative of this vector, pMorph20-C-gIII-hag2-LHC, contains a unique *Eco*RI site at the 3' end of the scFv while the second site (between the ompA signal sequence and the gIII ORF) was deleted via silent PCR mutagenesis. This construct allows the cloning of scFvs or scFv pools via the unique *Sph*I and *Eco*RI sites.

Attachment of scFvs to phage coat proteins via disulfide bonds

Phage for biopanning applications can be produced using helper phage VCSM13 following standard protocols (Kay et al., 1996). In addition to helper phage proteins, engineered phage coat protein and soluble modified scFv were co-expressed from the one- or two-vector systems described above. To demonstrate that the scFvs attach to the engineered phage coat proteins via disulfide bridges and are incorporated into phage particles, scFv displaying phages were run on SDS PAGE under non-reducing and reducing conditions. Western blot analysis was performed with anti-pIII and anti-Flag M1 antisera.

Phages were produced according to standard procedures using helper phage VCSM13 (Kay et al., 1996). Phages were pre-incubated in PBS with 5 mM DTT or without DTT (reducing and non-reducing conditions, respectively) for 30 minutes at room temperature before adding SDS loading buffer lacking reducing agents such as DTT or β-mercaptoethanol. 1 - 5 x 10^{10} phages per lane were run on a 4-15% SDS PAGE (BioRad) and blotted onto PVDF membranes. For the anti-pIII Western blot, the membrane was blocked in MPBST (PBS buffer containing 5% milk powder and 0.05% Tween20) and developed with mouse anti-pIII (1:250 dilution; Mobitec) as primary antibody, anti-mouse-IgG-AP conjugate (1:10000 dilution; SIGMA) as secondary antibody and BCIP/NPT tablets (SIGMA) as substrate. For the anti-Flag M1 Western blot, the membrane was blocked in MTBST- $CaCl_2$ (TBS buffer containing 5% milk powder, 0.05% Tween20 and 1 mM $CaCl_2$) and developed with mouse anti-Flag M1 (1:5000 dilution; Sigma) as primary antibody, anti-mouse-IgG-AP conjugate (1:10000 dilution; SIGMA) as secondary antibody and BCIP/NPT tablets (SIGMA) as substrate.

Specific bands migrating at the height expected for the scFv linked to the full length pIII could be shown both for the one- and two-vector system. This signal can only be seen under non-reducing conditions and disappears under DTT indicating that pIII and scFv are linked via disulfide bonds (scFv-S-S-pIII). As an example for the two-vector system an anti-Flag M1 and anti-pIII Western blot for scFv MacI-5 is shown in Figure 8. When the scFv without additional cysteines (pMorph7x-MacI-5-LH) is expressed, only free scFv sticking to phages can be detected in the anti-Flag M1 Western blot (lane 8, Figure 8A). When an additional cysteine is added to the scFv (pMorphX7-MacI-5-LHC), those bands can hardly be seen and a band migrating at the height of scFv dimers (scFv-S-S-scFv and/or $(scFv\text{-}SH)_2$) (and an unknown additional band (scFv-S-SX)) appear (lane 7, Figure 8A). When the engineered scFvs are co-expressed with an engineered pIII containing an additional cysteine at the N-terminus (pMorphX7-MacI-5-LHC and pBR-C-gIII) the signals shift to a molecular weight corresponding to scFv-pIII heterodimers (scFv-S-S-pIII) (lane 6, Figure 8A). As expected, this scFv-S-S-pIII signal cannot be seen when non-engineered scFvs are co-expressed with the engineered pIII (pMorphX7-MacI-5-LH and pBR-C-gIII), although similar numbers of phage particles are loaded in each lane (lane 5, Figure 8A). In the presence of reducing agents, the predominant signals are obtained from free scFvs for all expression systems (lanes 1 – 4, Figure 8A). In the anti-pIII Western blot, free protein III (pIII-SH and/or pIII) can be seen for all expression systems both under reducing and non-reducing conditions (lanes 1 – 8, Figure 8B). Specific bands migrating at the height expected for disulfide bonded protein III dimers (pIII-S-S-pIII) can only be detected under non-reducing conditions when engineered protein III is expressed (lanes 5 and 6 of Figure 8B). Only when both engineered scFv and engineered protein III are co-expressed an additional band migrating at the height of a disulfide-linked scFv and protein III (scFv-S-S-pIII) appears in addition to the disulfide bonded protein III dimers (lane 6, Figure 8B). This band corresponds in size to the scFv-S-S-pIII signal detected in the anti-Flag M1 Western (c.f. lane 6, Figure 8A) and is DTT sensitive (c.f. lane 2, Figure 8A). DTT sensitive bands migrating at the height of disulfide-linked scFv and protein III and being detected both with anti-Flag M1 and anti-pIII antisera were also observed when engineered scFv and engineered pIII were co-expressed from the same phagemid (pMorph18-C-pIII-scFv-LHC). As an example for this one-vector system an anti-Flag M1 and anti-pIII Western blot for scFv hag2 and anti-pIII Western blots for scFvs AB1.1 and MacI-5 are shown in Figures 9A and 9B, respectively.

Functionality of scFvs displayed on engineered phages

To show that the displayed scFvs are functional with respect to recognition of the specific antigen, phage ELISAs were performed. The analysis was done for the HuCAL scFvs MacI-5 and hag2. For the two-vector system, pMorphX7-LHC was co-transformed with pBR-C-gIII, pBR-C-gIIICT, pUC-C-gIII and pUC-C-gIIICT, respectively. Three different one-vector constructs were analysed, namely pMorph18-C-gIII-scFv-LHC, pMorph18-C-gIIICT-scFv-LHC and pMorph18-C-gIX-scFv-LHC. To demonstrate that the scFvs attach to the engineered phage coat proteins via disulfide bonds, phage ELISAs were performed both under non-reducing and reducing conditions.

Phages were produced according to standard procedures using helper phage VCSM13 and phage titers were determined (Kay et al., 1996). Specific antigen or control antigen (BSA, Sigma #A7906) was coated for 12 hours at 4 °C at an amount of 5 µg/well in PBS to Nunc Maxisorp microtiter plates (# 442404) and blocked with PBS containing 5% skimmed milk powder for 2 h. Phages were pre-incubated in PBS containing 2.5% skimmed milk powder, 0.05% Tween 20, as well as 5 mM DTT, where applicable, for 2 h at room temperature before they were applied to the ELISA well coated with antigen at a concentration range between 6.4 x 10^6 and 1 x 10^{11} phages per well. After binding for 1 h at RT, unspecifically bound phages were washed away with PBS containing 0.05% Tween 20 and bound phages were detected in ELISA using an anti-M13-HRP conjugate (Amersham Pharmacia Biotech #27-9421-01) and BM blue soluble (Boehringer Mannheim #1484281). Absorbance at 370 nm was measured. ELISA signals obtained with the specific antigen were compared to those with the control antigen. Specific binding of scFv displaying phages to antigen could be shown for the C-gIII, C-gIIICT and C-gIX constructs in the one-vector format. C-gIII and C-gIIICT were also tested and shown to work in both two-vector systems. As an example four such ELISAs for scFv MacI-5 are presented in Figures 10-13. In all cases where phage coat proteins are engineered with an additional cysteine residue, ELISA signals are significantly increased compared to the pMorphX7-LHC signals where only the scFv carries an additional cysteine. When 5 mM DTT was added to the phages prior to antigen binding during the pre-incubation step, the ELISA signal was decreased to almost background levels for all three engineered phage coat

constructs as well as the non-engineered pMorphX7-LHC phages while DTT had no major effect on the conventional display phages (pMorph13; Figure 13). This shows that for both the non-engineered and engineered phages disulfide bonds are essential for the functional display of scFvs on phages and thus for the specific binding of scFv displaying phages to antigen.

Enrichment of engineered phages displaying scFv in "doped library" experiments

To prove that engineered phages displaying scFvs can be enriched on specific antigen, a "doped library" experiment was performed: specific phages were mixed with a high excess of unspecific phages and three rounds of panning on specific antigen were performed The enrichment for specific phages was determined after each round. The analysis was done for the two HuCAL scFvs hag2 and MacI-5 in the pMorph18-C-gIII-scFv-LHC one-vector system.

pMorph18-C-gIII-hag2-LHC and pMorph18-C-gIII-MacI-5-LHC derived phages were mixed at ratios of $1:10^5$ (pMorph18-C-gIII-hag2-LHC panning) as well as $10^5:1$ (pMorph18-C-gIII-MacI-5-LHC panning). Three rounds of panning were performed on the hag2 and MacI-5 specific antigen, respectively. Phages were prepared by standard procedure and pre-blocked by mixing 1:1 with PBSTM (PBS, 5% skimmed milk powder, 0.1% Tween20) and incubation for 2 h at RT. Wells of a Nunc Maxisorp plate (#442404) were coated with specific antigen (as well as BSA) at a concentration of 5 µg/well in PBS overnight at 4°C, and subsequently blocked with 400 µl PBSM (PBS, 5% skimmed milk powder) for 2 h at RT. For the first round, 10^{10} pre-blocked phages were applied per well and incubated for 1 h at RT on a microtiter plate shaker. Phage solution was removed and wells were washed 3 times with PBST (PBS, 0.05% Tween20) and 3 times with PBS. Bound phages were eluted with 100 mM triethylamine according to standard protocols and used for infection of TG1 cells. In addition, residual phages were eluted by direct infection of TG1 cells added to the wells. After each round of panning on specific antigen, the ratio of specific to unspecific phages was determined by analysing at least 91 independent infected cells via PCR. The PCR was performed according to standard protocols using single colonies as source of template and oligonucleotides specific for VH CDR3 and VL CDR3 of each scFv as primers. After 2 rounds of panning, ~ 0% positive clones (0 out of 93 clones analysed) were obtained for the pMorph18-C-gIII-hag2-LHC panning and ~ 3% positive clones (3 out of 91 clones analysed)

were obtained for the pMorph18-C-gIII-MacI-5-LHC panning. After 3 rounds of panning, the specific clones were enriched to ~ 79% (92 out of 117 clones analysed) for the pMorph18-C-gIII-hag2-LHC panning and to ~ 100% (229 out of 229 clones analysed) for the pMorph18-C-gIII-MacI-5-LHC panning.

Enrichment of engineered phages displaying scFv in pannings of pre-selected pools

To prove that engineered phages displaying scFvs can be selected out of a diverse pool, pannings of pre-selected libraries were performed. Pools after one round of conventional panning were subcloned into the engineered one-vector format and panning was continued for up to three further rounds (cys-display pannings).

Pannings were performed against the following antigens: (i) ICAM1 comprising the extracellular part of mature ICAM1 (amino acids 1-454) plus amino acids CGRDYKDDDK*HHHHHH* containing the M2-Flag and the 6x histidine tag. (ii) N1-MacI comprising aa 1-82 of mature gene III protein of phage M13 containing an additional methionine residue at the N-terminus plus a short linker at the C-terminus (N1), fused to a polypeptide containing amino acids 149-353 of human CR-3 alpha chain (SWISS-PROT entry P11215) plus the C-terminal sequence IEGR*HHHHHH* which includes the 6x histidine tag; and (iii) N1-Np50 comprising N1 fused to a polypeptide containing amino acids 2-366 of human NFκB p50 plus amino acids EFS*HHHHHH* which include the 6x histidine tag. Expression vectors for N1-MacI and N1-Np50 are based on vector pTFT74 (Freund et al., 1993) (complete vector sequence of pTFT74-N1-hag-HIPM given in Fig. 2). Expression, purification and refolding was done as described (Krebber, 1996; Krebber et al., 1997).

Initially, one round of conventional panning of the antibody library HuCAL-scFv (WO 97/08320; Knappik et al., 2000) was performed according to standard protocols. Briefly, wells of Maxisorp microtiterplates (Nunc; #442404) were coated with the respective antigen dissolved in PBS and blocked with 5 % skimmed milk powder in PBS. 1–5 x 10^{12} HuCAL-scFv phage were added for 1 h at 20°C. After several washing steps with PBST (PBS, 0.05% Tween20) and PBS, bound phage were eluted either with 100 mM triethylamine or 100 mM glycine pH 2.2, immediately neutralised with 1 M Tris/HCl pH 7.0 and used for infection of TG1 cells. In addition, residual phages were eluted by direct infection of TG1 cells added to

the wells. Pannings against N1-Np50 used the complete HuCAL-scFv library (κ and λ pools combined), in pannings against N1-MacI κ and λ light chain pools were kept separated. Against ICAM1 one round of conventional panning of the λ light chain part of HuCAL-scFv was performed and subsequently the selected heavy chains again combined with the complete library of λ light chains. The resulting light chain optimised library had a diversity of $1.4x\ 10^7$.

The scFvs of the respective pools were subcloned into vector pMorph20-C-gIII-scFv-LHC (one-vector format) via the unique *Sph*I and *Eco*RI sites. Subsequently, three rounds of cys-display panning were performed. Phages were prepared by standard procedure and pre-blocked by mixing 1:1 with PBSTM (PBS, 5% skimmed milk powder, 0.1% Tween20) and incubated for 2 hrs at RT. Wells of a Nunc Maxisorp plate (#442404) were coated with specific antigens at a concentration of 5 µg/well in PBS overnight at 4°C, and subsequently blocked with 400 µl PBSM (PBS, 5% skimmed milk powder) for 2 hrs at RT. For each round of cys-display panning, between $1x\ 10^{10}$ and $4.5x\ 10^{11}$ pre-blocked phages were applied per well and incubated for 1 h at RT on a microtiter plate shaker. Phage solution was removed and wells were washed with PBST (PBS, 0.05% Tween20) and PBS with increasing stringency. The 1st round was washed 3x quick and 2x 5 min with PBST and PBS, respectively, the 2nd round 1x quick and 4x 5 min with PBST and PBS, respectively, and the 3rd round 10x quick and 5x 5 min with PBST and PBS, respectively. Bound phages were eluted with 100 mM triethylamine according to standard protocols and used for infection of TG1 cells. In addition, residual phages were eluted by direct infection of TG1 cells added to the wells.

After each round of panning the number of antigen specific phages was determined in an ELISA. N1-MacI, N1-Np50 and ICAM-Strep (comprising amino acids 1-455 of mature ICAM1 plus SA*WSHPQFEK* containing the Strep-tag II) were used as antigens, respectively. To ensure high level expression the selected scFvs were subcloned into expression vector pMorphX7-FS (Table 1). Subcloning was done in two steps. First the scFv fragments were isolated from pMorph20-C-gIII-scFv-LHC via *Afl*II and *Eco*RI, then the fragments were re-digested with *Sph*I and cloned into the *Eco*RI/*Sph*I digested pMorphX7-FS vector. This procedure ensured that only scFvs from vector pMorph20-C-gIII-scFv-LHC were subcloned and excluded any contamination with scFvs from a conventional display or expression vector. Expression of the scFvs and their testing in ELISA against the respective antigens was done according to standard procedures. Clones which showed a signal of at least 3x above

background in ELISA were considered positive. The results are summarised in Table 5. To prove that the selected scFvs bind strongly and specifically to their respective antigen several positive clones after 2 rounds of cys-display panning were selected and re-tested in quadruplicates in a specificity ELISA on six different antigens (Figures 14 & 15). Enrichment of antigen-specific binders could clearly be demonstrated. Already after two rounds of cys-display panning of the pre-selected pools against N1-MacI, N1-Np50 and ICAM1 between 80 % and 97 % of the tested clones were positive in ELISA. The affinity of some of the selected scFvs was determined in Biacore and Kd values in the range of 1 nM to 2.2 μM were determined. These results are similar to the enrichment factors and affinities obtained in a conventional panning of the respective pools performed in parallel. Some of the scFvs were selected independently via cys-display as well as conventional panning.

Elution of engineered phages displaying scFv via reducing agents

When screening phage display libraries in biopanning the problem remains how to best recover phages which have bound to the desired target. Normally, this is achieved by elution with appropriate buffers, either by using a pH- or salt gradient, or by specific elution using soluble target. However, the most interesting binders which bind with high affinity to the target might be lost by that approach. One option with engineered cys-display phages is that the complexes of target and specific bacteriophages can be treated with reducing agents, e.g. by incubation with DTT, to cleave the disulfide bond between scFv and phage coat protein and to recover the specific bacteriophage particles.

Pannings of pre-selected pools against N1-MacI were performed according to the protocol described above. Phages were eluted either according to the standard protocol with 100 mM triethylamine and a direct infection of TG1 cells by residual phages, or by incubation of the wells with 20 mM DTT in Tris buffer pH 8.0 for 10 min. After each round of panning the pool of selected scFvs was subcloned into expression vector pMorphX7-FS according to the two step procedure described above, and the number of N1-MacI specific scFvs was determined in ELISA. To prove that the selected scFvs bind strongly and specifically to their respective antigen several positive clones were selected and re-tested in triplicates in a specificity ELISA. Enrichment of antigen-specific binders could clearly be demonstrated for both elution

procedures. After two rounds of panning of the MacI κ-pool and the MacI λ-pool a two-fold and five-fold, respectively, higher number of ELISA positive clones was obtained for elution with reducing agents compared to conventional elution.

Example 2.2: Display of Fabs

Example 2.1 shows that functional single chain fragments can be displayed on engineered phages via disulfide bonds. In the following we will describe experiments which show that the same is true for Fabs. The cysteine was engineered at different positions of the Fab antibody fragment. These Fabs were co-expressed in the same bacterial cell together with engineered full length pIII based on a two-vector system.

Construction of vectors expressing Fabs and engineered pIII

Heavy and light chains of the Fab fragment were expressed from a dicistronic phagemid under control of the lac promotor/operator region. The first expression cassette comprises the signal sequence ompA and the variable and constant domain of the light chain, the second expression cassette comprises the signal sequence phoA and the variable and constant domain of the heavy chain. Heavy and light chain are not linked via a disulfide bond. Modules containing the engineered cysteine were located at the C-terminus of either the light or the heavy chain. Several constructs differing in the amino acid composition of the modules were compared and are summarised in Table 6. As an example the complete vector sequence of pMorphX10-Fab-VL-LHC-VH-FS coding for the modified Fab MacI-5 and the respective vector map are given in Figure 16a+b.

Two different plasmids were used for expression of full length pIII. Plasmid pBR-C-gIII was already described above. The respective expression cassette comprises the signal sequence ompA, amino acids DYCDIEF and the pIII ORF under control of the lactose promotor/ operator region (Table 3, Figure 6). Alternatively, plasmid pBAD-SS-C-gIII was used. Here the respective expression cassette comprises the signal sequence of pIII, amino acids TMACDIEF and the pIII ORF under control of the arabinose promotor/operator region (Table 3). For construction of pBAD-SS-C-gIII the fragment coding for the engineered cysteine plus

pIII was amplified from pUC-C-gIII via PCR introducing the restriction sites *Nco*I and *Hin*dIII and cloned into the commercially available vector pBAD/gIII A (Invitrogen). The plasmids pBR-C-gIII or pBAD-SS-C-gIII were co-transformed with the respective pMorphX10-Fab phagemids expressing the modified Fab into *E.coli* TG1 selecting for both antibiotic markers.

Description of the Fab – antigen interactions

Three different Fabs all deriving from a human combinatorial antibody library (HuCAL; WO 97/08320; Knappik et al., 2000) were used for evaluation of Fab display on engineered phage. The HuCAL VH and VL consensus genes (described in WO 97/08320), and the CDR3 sequences of the Fabs are given in Table 2. Fab MacI-5 is derived from the scFv MacI-5 described above and was converted into the Fab format (complete vector map of pMorphX10-Fab-MacI5-VL-LHC-VH-FS is given in Figure 16a). Fabs MacI-A8 and ICAM1-C8 were isolated directly from one of the HuCAL-Fab libraries. Clone MacI-A8 was selected against antigen MacI-Strep, which comprises an N-terminal methionine, amino acids 149-353 of human CR-3 alpha chain (SWISS-PROT entry P11215) and amino acids SA*WSHPQFEK* which include the Strep-tag II (Schmidt et al., 1996). Expression and purification were done according to Schmidt & Skerra (1994). N1-MacI was used as corresponding antigen for ELISAs. N1-MacI is described above, and comprises an N-terminal methionine, amino acids 1-82 of mature gene III protein of phage M13 plus a short linker (N1), amino acids 149-353 of human CR-3 alpha chain (SWISS-PROT entry P11215) and amino acids IEGR*HHHHHH* which include the 6x histidine tag. Clone ICAM1-C8 was selected against antigen ICAM1 described above, which comprises the extracellular part of mature ICAM1 (amino acids 1-454) plus amino acids CGRDYKDDDK*HHHHHH* containing the M2-Flag and the 6x histidine tags. The same antigen was used for ELISA assays as well as in the doped library experiment.

Attachment of Fabs to phage coat proteins via disulfide bonds

To demonstrate that the Fabs attach to pIII via disulfide bridges and are incorporated into phage particles, the respective phages were run on SDS PAGE under non-reducing and reducing conditions. Western blot analyses were performed with antibodies detecting pIII, the

heavy chain, the lambda light and the kappa light chain, respectively. All constructs described in table 6 were analysed and the results are shown for pMorphX10-ICAM1C8-VL-LHC-VH-FS plus pBAD-SS-C-gIII and pMorphX10-MacIA8-VL-LHC-VH-MS plus pBAD-SS-C-gIII as an example in Figures 17 and 18.

Phages were produced using helper phage VCSM13 following standard protocols (Kay et al., 1996). In addition to helper phage proteins, engineered phage coat protein and soluble modified Fab were co-expressed from the two-vector system. Phages were pre-incubated in PBS with or without 20 mM DTT (reducing and non-reducing conditions, respectively) for 1 h at room temperature before adding SDS loading buffer lacking reducing agents such as DTT or β-mercaptoethanol. 1x 10^{10} phages per lane were run on a 12 % SDS PAGE (BioRad) and blotted onto nitrocellulose membranes (Schleicher & Schuell). For the anti-pIII Western blot, the membrane was blocked in MTBST (50 mM Tris buffer pH 7.4, containing 5% milk powder and 0.05% Tween20) and developed with mouse anti-pIII (1:250 dilution; Mobitec) as primary antibody, anti-mouse-IgG-HRP conjugate (1:5000 dilution; SIGMA) as secondary antibody and BM Blue POD precipitating (Roche #1442066) as substrate. For the detection of the heavy chain, kappa light and lambda light chain the primary antibodies anti-Fd (1:5000 dilution; The binding site PC075), anti-human kappa (1:5000 dilution; Sigma K-4377) and anti-human lambda (1:500 dilution, Sigma L-6522) were used, respectively.

In the anti-pIII Western blots, free protein III (SH-pIII and/or pIII) can be detected for all expression systems both under reducing and non-reducing conditions (Figures 17 and 18). When both engineered Fab and engineered protein III are co-expressed a signal migrating at the height of a hetero-dimer of light chain and protein III (VL-CL-SS-pIII) appears under non-reducing conditions. In addition, a band migrating at the height expected for disulfide bonded protein III dimers (pIII-SS-pIII) can be seen (lanes 11 & 12, Figures 17,18). Both hetero- and homo-dimers disappear when the samples are treated with DTT (lanes 5 & 6, Figures 17 and 18) or when modified Fabs are coexpressed with non-engineered pIII (lanes 3, 4, 9 & 10, Figures 17 and 18). The hetero-dimer in this case of light chain linked to the full length pIII could also be detected with anti-light chain antibodies in non-reducing gels but was absent under reducing conditions. In addition, a band migrating at the height expected for the homo-dimer of the light chain (VL-CL-SS-VL-CL) was detectable (data not shown). Similar results were obtained for all constructs described in Table 6, and no significant difference between

vectors pBR-C-gIII and pBAD-SS-C-gIII for supply of engineered pIII was detected (data not shown).

Functionality of Fabs displayed on engineered phages

To show that the displayed Fabs are functional with respect to recognition of the specific antigen phage ELISAs were performed. The analysis was done for the HuCAL Fabs MacI-5, MacI-A8 and ICAM1-C8. All formats differing in the position of cysteine at the Fab were compared (Table 6). To demonstrate that the Fabs attach to the engineered phage coat proteins via disulfide bonds, phage ELISAs were performed both under non-reducing and reducing conditions.

The respective phagemids expressing the modified Fab were co-transformed with pBR-C-gIII and phage production was performed under standard conditions (Kay et al., 1996). Conventional Fab display phages (pMorph18-Fab) served as positive control, a phagemid expression vector for expression of non-engineered Fab (pMorphX9-Fab-FS) served as negative control. Specific antigen or control antigen (BSA, Sigma #A7906) was coated for 12 hours at 4 °C at an amount of 5 µg/well in PBS to Nunc Maxisorp microtiter plates (# 442404) and blocked with PBS containing 5 % skimmed milk powder, 0.05 % Tween 20 for 1 h. Phages were pre-incubated in PBS containing 5 % skimmed milk powder, 0.05 % Tween 20, and 10 mM DTT where applicable for 1 h at room temperature before they were applied to the ELISA well coated with antigen at a concentration range between $1x\ 10^8$ and $1\ x\ 10^{10}$ phages per well. After binding for 1 h at RT, unspecifically bound phages were washed away with PBS containing 0.05% Tween 20 and PBS. Bound phages were detected in ELISA using an anti-M13-HRP conjugate (Amersham Pharmacia Biotech #27-9421-01) and BM blue soluble (Roche #1484281). Absorbance at 370 nm was measured. ELISA signals obtained with the specific antigen were compared to those with the control. Up to three independent phage preparations were analysed and mean values are given in Figures 19 to 22.

For all different two-vector formats specific binding of Fab displaying phages to antigen could be demonstrated (Figures 19-21, lanes 1-4). For Fab MacI-5 no significant difference between the four formats was detected (Figure 19), while construct pMorphX10-Fab-VL-LHC-VH-FS

showed reproducibly best results for Fab MacI-A8 and ICAM1-C8 (Figures 20 and 21). When 10 mM DTT was added to the phages prior to antigen binding during the pre-incubation step, the ELISA signal was decreased to almost background levels for all cys-display phages while DTT had no major effect on conventional display phages (pMorph18-Fab) (shown for Fab MacI-5 in Figure 22). This shows that disulfide bonds are essential for the functional display of Fabs on phages and thus for the specific binding of Fab displaying phages to antigen.

Enrichment of engineered phages displaying Fabs in doped library experiments

To prove that engineered phages displaying Fabs can be enriched on specific antigen, a "doped library"experiment was performed: specific phages were mixed with a high excess of unspecific phages and three rounds of panning on specific antigen were performed. The enrichment for specific phages was determined after each round. The analysis was done for the HuCAL Fab ICAM1-C8 in the two vector system pMorphX10-Fab-VL-LHC-VH-FS plus pBAD-SS-C-gIII.

Engineered phages displaying ICAM1-C8 and MacI-A8 were mixed at ratios of $1:10^5$. Three rounds of panning were performed on the ICAM1 antigen. Phages were prepared by standard procedure, pre-blocked by mixing 1:1 with PBSTM (PBS, 5 % skimmed milk powder, 0.1 % Tween 20) and incubated for 2 hrs at RT. Wells of a Nunc Maxisorp plate (#442404) were coated with specific antigen at a concentration of 5 µg/well in PBS overnight at 4°C, and subsequently blocked with 400 µl PBSM (PBS, 5 % skimmed milk powder) for 2 hrs at RT. For the first round, 10^{11} pre-blocked phages were applied per well and incubated for 1 h at RT on a microtiter plate shaker. Phage solution was removed and wells were washed 3 times with PBST (PBS, 0.05 % Tween 20; 1x quick, 2x 5 min) and 3 times with PBS (1x quick, 2x 5 min). Bound phages were eluted with 100 mM triethylamine according to standard protocols. In addition, residual phages were eluted by direct infection of cells added to the wells. As a direct infection of TG1 cells harbouring pBAD-SS-C-gIII was not efficient enough, eluted phages were used for infection of TG1 cells, amplified and than used for infection of TG1 cells harbouring pBAD-SS-C-gIII. Thus the two-vector system was restored and the next round of panning was performed. While no difference between the two plasmids for expression of engineered pIII (pBR-C-gIII and pBAD-SS-C-gIII) was observed with respect to phage ELISA and WB, infection of TG1 cells harbouring pBR-C-gIII was not as

efficient as infection of TG1 cells harbouring pBAD-SS-C-gIII. After each round of panning the ratio of specific to unspecific phages was determined by analysing at least 92 independent infected cells via PCR. The PCR was performed according to standard protocols using single colonies as source of template and oligonucleotides specific for the lambda light chain (priming in framework 4), the kappa light chain (priming in framework 3) and a vector sequence upstream of the Fab fragment (commercial M13-rev primer, NEB) as primers. Fragments of roughly 420 bp length were expected for lambda Fabs (ICAM1-C8) and 290 bp for kappa Fabs (MacI-A8). After 2 rounds of panning, 61 % positive clones (57 out of 93 clones analysed) were obtained, which could be enriched to 100 % (92 out of 92 clones analysed) after the third round.

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Table 1: Amino acid sequence of ORF modules between the *Eco*RI and *Hin*dIII sites of vectors pMorphX7-hag2-FS, pMorphX7-hag2-LH, pMorphX7-hag2-LCH and pMorphX7-hag2-LHC

Construct	*Eco*RI	Module 1	*Asc*I	Module 2	*Hin*dIII
pMorphX7-FS	EF	DYKDDDDK	GAP	WSHPQFEK-stop	stop
pMorphX7-LH	EF	PGGSG	GAP	HHHHHH-stop	stop
pMorphX7-LCH	EF	PGGSG	GAP	CHHHHHH-stop	stop
pMorphX7-LHC	EF	PGGSG	GAP	HHHHHHC-stop	stop

Table 2: Amino acid sequence of HuCAL scFvs and HuCAL Fabs*

scFv	antigen	VH	VH CDR3	VL	VL CDR3
hag2	peptide of influenza virus hemagglutinine (*CAG*PYDVPDYASLRS*H H*)	VH3	RSGAYDY	Vκ4	QQYSSFPL
AB1.1	12 amino acid peptide	VH3	10 amino acid residues	Vλ1	9 amino acid residues
MacI-5	fragment of human CR-3 alpha chain	VH2	FDPFFDSFFDY	Vλ1	QSYDQNALVE
MacI-A8	fragment of human CR-3 alpha chain	VH3	HGYRKYYTDM FDV	Vκ1	HQVYSTSP
ICAM1-C8	human ICAM1	VH2	FPYTYHGFMD N	Vλ3	QSYDSGNL

* details are given in the Examples

Table 3: Amino acid sequence of engineered phage coat proteins of vector pBR-C-gIII and derivatives

Construct	Signal Sequence		*Eco*RV-*Eco*RI	sequence	*Hin*dIII
pUC-C-gIII pBR-C-gIII	MKKTAIAIAVAL AGFATVAQA (ompA)	DY**C**	DI EF	AETVESCLAKPHTENSFTNVWKDD KTLDRYANYEGCLWNATGVVVCT GDETQCYGTWVPIGLAIPENEGGGS EGGGSEGGGSEGGGTKPPEYGDTPI PGYTYINPLDGTYPPGTEQNPANPN PSLEESQPLNTFMFQNNRFRNRQGA LTVYTGTVTQGTDPVKTYYQYTPV SSKAMYDAYWNGKFRDCAFHSGF NEDPFVCEYQGQSSDLPQPPVNAG GGSGGGSGGGSEGGGSEGGGSEGG GSEGGGSGGGSGSGDFDYEKMAN ANKGAMTENADENALQSDAKGKL DSVATDYGAAIDGFIGDVSGLANG NGATGDFAGSNSQMAQVGDGDNS PLMNNFRQYLPSLPQSVECRPYVFG AGKPYEFSIDCDKINLFRGVFAFLL YVATFMYVFSTFANILRNKES	stop
pUC-C-gIIICT pBR-C-gIIICT	MKKTAIAIAVAL AGFATVAQA (ompA)	DY**C**	DI EF	NAGGGSGGGSGGGSEGGGSEGGGS EGGGSEGGGSGGGSGSGDFDYEK MANANKGAMTENADENALQSDAK GKLDSVATDYGAAIDGFIGDVSGL ANGNGATGDFAGSNSQMAQVGDG DNSPLMNNFRQYLPSLPQSVECRPF VFGAGKPYEFSIDCDKINLFRGVFA FLLYVATFMYVFSTFANILRNKES	stop
pBAD-SS-C-gIII	MKKLLFAIPLVV PFYSHS (gIII)	TMA **C** *Nco*I (*Sty*I) / *Sph*I	DI EF	AETVESCLAKPHTENSFTNVWKDD KTLDRYANYEGCLWNATGVVVCT GDETQCYGTWVPIGLAIPENEGGGS EGGGSEGGGSEGGGTKPPEYGDTPI PGYTYINPLDGTYPPGTEQNPANPN PSLEESQPLNTFMFQNNRFRNRQGA LTVYTGTVTQGTDPVKTYYQYTPV SSKAMYDAYWNGKFRDCAFHSGF NEDPFVCEYQGQSSDLPQPPVNAG GGSGGGSGGGSEGGGSEGGGSEGG GSEGGGSGGGSGSGDFDYEKMAN ANKGAMTENADENALQSDAKGKL DSVATDYGAAIDGFIGDVSGLANG NGATGDFAGSNSQMAQVGDGDNS PLMNNFRQYLPSLPQSVECRPYVFG AGKPYEFSIDCDKINLFRGVFAFLL YVATFMYVFSTFANILRNKES	stop

The engineered Cys is written in bold
Sequence of wild type phage coat proteins is underlined

Table 4: Amino acid sequence of engineered phage coat proteins of vector pMorph18-C-gIII-scFv-LHC and derivatives

Construct	OmpA Signal Sequence		*Eco*RV -*Eco*RI	sequence	*Stu*I
pMorph18-C-gIII-scFv-LHC	MKKTAIAIAVAL AGFATVAQA	DY **C**	DI EF	AETVESCLAKPHTENSFTNVWKD DKTLDRYANYEGCLWNATGVVV CTGDETQCYGTWVPIGLAIPENEG GGSEGGGSEGGGSEGGGTKPPEY GDTPIPGYTYINPLDGTYPPGTEQN PANPNPSLEESQPLNTFMFQNNRF RNRQGALTVYTGTVTQGTDPVKT YYQYTPVSSKAMYDAYWNGKFR DCAFHSGFNEDPFVCEYQGQSSDL PQPPVNAGGGSGGGSGGGSEGGG SEGGGSEGGGSEGGGSGGGSGSG DFDYEKMANANKGAMTENADEN ALQSDAKGKLDSVATDYGAAIDG FIGDVSGLANGNGATGDFAGSNS QMAQVGDGDNSPLMNNFRQYLP SLPQSVECRPYVFGAGKPYEFSID CDKINLFRGVFAFLLYVATFMYVF STFANILRNKES	stop
pMorph18-C-gIIICT-scFv-LHC	MKKTAIAIAVAL AGFATVAQA	DY **C**	DI EF	NAGGGSGGGSGGGSEGGGSEGGG SEGGGSEGGGSGGGSGSGDFDYE KMANANKGAMTENADENALQSD AKGKLDSVATDYGAAIDGFIGDV SGLANGNGATGDFAGSNSQMAQ VGDGDNSPLMNNFRQYLPSLPQS VECRPFVFGAGKPYEFSIDCDKINL FRGVFAFLLYVATFMYVFSTFANI LRNKES	stop
pMorph18-C-gIX-scFv-LHC	MKKTAIAIAVAL AGFATVAQA	DY **C**	DI EF	GGGGSMSVLVYSFASFVLGWCLR SGITYFTRLMETSS	stop

The engineered Cys is written in bold
Sequence of wild type phage coat proteins is underlined

Table 5: Cys-display panning of pre-selected pools

Preselected Pool	# of clones[a]	# of positives[b]	Panning Format	round 1	round 2	round 3
N1-MacI κ chains	2x 10^5	3/186 = 2 %	Cys-display	78/279 = 28 %	89/93 = 96 %	92/93 = 99 %
			conventional	10/93 = 11 %	71/93 = 76 %	nd
N1-MacI λ chains	4x10^4	4/186 = 2 %	Cys-display	72/279 = 26 %	90/93 = 97 %	90/93 = 97 %
			conventional	34/93 = 37 %	87/93 = 94 %	nd
N1-Np50	5x 10^4	0/186 = 0 %	Cys-display	17/93 = 18 %	244/279 = 87 %	nd
			conventional	51/93 = 55 %	86/93 = 92 %	nd
ICAM1	1.4x 10^7	nd	Cys-display	4/186 = 2 %	149/186 = 80 %	nd

nd: not determined

[a] N1-MacI, N1-Np50: number of clones after one round of conventional panning; ICAM1: diversity of the light chain optimised pool.

[b] number of ELISA positives of the respective pre-selected pools.

Table 6: Amino acid sequence of modules of engineered Fab fragment

Construct	Module at the light chain		Module at the heavy chain	
	elements	amino acids	elements	amino acids
pMorphX10-Fab-VL-LHC-VH-FS	linker-histidine tag-cysteine	SPGGSG-GAP-HHHHHH-**C**-stop	linker Flag tag-linker Strep-tag II	EF-DYKDDDDK-GAP-WSHPQFEK-stop
pMorphX10-Fab-VL-LHC-VH-MS	linker-histidine tag-cysteine	SPGGSG-GAP-HHHHHH-**C**-stop	linker-myc tag-linker-Strep-tag II	EF-EQKLISEEDLN-GAP-WSHPQFEK-stop
pMorphX10-Fab-VL-C-VH-FS	cysteine	deletion of A-**C**-stop (κ-chains) **C**S-stop (λ-chains)	linker-Flag tag-linker-Strep-tag II	EF-DYKDDDDK-GAP-WSHPQFEK-stop
pMorphX10-Fab-VL-C-VH-MS	cysteine	deletion of A-**C**-stop (κ-chains) **C**S-stop (λ-chains)	linker-myc tag-linker-Strep-tag II	EF-EQKLISEEDLN-GAP-WSHPQFEK-stop
pMorphX10-Fab-VL-VH-LHC	-	-	linker-histidine tag-cysteine	EF-PGGSG-GAP-HHHHHH-**C**-stop
pMorphX10-Fab-VL-VH-CFS	-	-	cysteine-linker-Flag tag-linker-Strep-tag II	**C**-EF-DYKDDDDK-GAP WSHPQFEK-stop
pMorphX10-Fab-VL-VH-CMS	-	-	cysteine-linker-myc tag-linker-Strep-tag II	**C**-EF-EQKLISEEDLN-GAP-WSHPQFEK-stop

The engineered cysteine is written in bold